(12) UK Patent Application (19) GB (11) 2 053 926 A

(21) Application No 8023262
(22) Date of filing 16 Jul 1980
(30) Priority data
(31) 7925485
(32) 20 Jul 1979
(33) United Kingdom (GB)
(43) Application published
11 Feb 1981
(51) INT CL³
C07G 7/00
(52) Domestic classification
C3H F2
(56) Documents cited
GB 1574414
GB 1540165
GB 1461528
C.A. *92* 124284q
C.A. *90* 83076m
C.A. *87* 196593]
(C.A. *89* 142885d
(US 4093612))
(58) Field of search
C3H
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# (54) Albumin extraction by affinity chromatography

(57) Albumin may be separated from mixtures such as blood, plasma or blood protein fractions by affinity chromatography on an immobilised triazine dye of high adsorptive capacity obtained by immobilisation with alkali metal hydroxide. The albumin is preferably adsorbed at pH 6—8 and at above 20°, more preferably 30—40°C and is eluted with a solution containing a carboxylic acid, normally as alkali metal salt. $C_4$—$C_{12}$ carboxylic acids, especially octanoic, are preferred. The albumin may subsequently be heat treated to improve its agglutination enhancing activity and shows a low tendency to rouleaux formation. Alternatively the separation may be used in conjunction with a plasmaphoresis process, the patient receiving his own albumin as well as red cells.

Certain of the chemical formulae appearing in the printed specification were submitted in formal form after the date of filing.

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SPECIFICATION

**Purification of albumin**

The invention relates to methods for separating albumin, especially human albumin, from mixtures containing same by affinity chromatography. The invention is especially applicable to albumin separation during plasmaphoresis.

Albumin is the major of a number of protein fractions occurring in blood plasma and serum. Separated albumin is itself a commercially important product and additionally albumin removal is an important step in isolation of other protein fractions or plasma or serum components such as enzymes. Traditional albumin separations are long and tedious and hence it has recently been proposed, for example in U.K. Patent 1,461,528, to separate albumin by affinity chromatography on triazine dyes or dye-dextran conjugates immobilised on agarose, polyacrylamide or acrylic resin supports.

These dyes are immobilised by the method described in, for example, U.S. Patent 4,016,149 and by Baird et al, Febs Letters, Vol. 70 (1976) page 61, wherein the dyes are bound to the matrices by substitution at the chloride group. Such binding is carried out in alkaline, sodium carbonate or bicarbonate buffered, media and since the dyes are designed for dyeing cellulose, the bound dye concentrations on non-cellulosic matrices are generally very low resulting in low protein binding capacity. The dye-dextran conjugates contain no free chlorine and are bound through amine groups on the dye, following cyanogen bromide activation of the support. However cyanogen bromide activation has serious disadvantages, especially for industrial use: the material is highly toxic and hence too dangerous to use on an industrial scale or in the preparation of pharmaceutical products; it produces amido carbonate and carbamate links with the matrix which are unstable and cause gradual loss of column activity in long-term use; and it activates all hydroxyl and amine groups on the dye resulting in dye-dye bonding and dye-bonding other than through the triazine group so that only a small proportion of the bound dye molecules are available for protein binding and their protein binding properties may differ. For example Travis and Pannell (Clin. Chem. Acta.) *49* pp 49—52, 1973) report that a Procion Blue H—B dextran conjugate bound to Sepharose ('Procion' and 'Sepharose' are Trade Marks) following cyanogen bromide activation will bind albumin whereas the dye itself similarly bound will not.

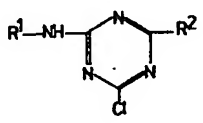
There is thus a need for affinity chromatography media with a high albumin binding capacity which may be prepared without the use of cyanogen bromide.

It has now been found that media prepared in accordance with the process of our copending U.K. Patent Application No. 3505/78 can meet this need and in combination with suitable eluting agents can provide a highly efficient process for separation of albumin.

The present invention therefore provides a process for the separation of albumin from mixture containing same comprising contacting said mixture with a suspension of an affinity chromatography medium to which the albumin will bind, washing said medium to remove unbound components of said mixture and eluting the albumin from said medium by washing with an eluting solution containing a carboxylic acid, preferably having 4 to 12 carbon atoms, wherein the chromatography medium is an immobilised triazine dye produced by reacting a protein-binding ligand material containing chlorotriazinyl groups with an aqueous suspension of a non-cellulosic matrix containing free hydroxy or amino groups in the presence of an alkali metal hydroxide at a pH of at least 8 and subsequently washing the resulting solid medium to remove unreacted dye.

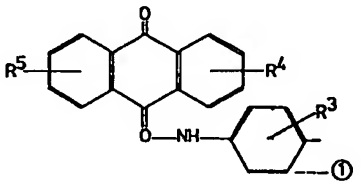
It is surprisingly found that the use of an alkali metal hydroxide in this reaction results in very much higher binding of the protein-binding ligand to the matrix than occurs with sodium carbonate or other bases under identical conditions of temperature, time and alkali concentration. In addition the present invention is capable of producing media showing tighter elution profiles under similar conditions than the media of the prior art.

The protein-binding ligand may be any mono or di-chloro triazinyl compounds commonly called "triazinyl dyes" such as those sold under the Trade Marks "Cibacron" and "Procion". These are normally triazinyl derivatives of sulphonated anthroquinones, thalocyanines or polyaromatic azo compounds. Such compounds have the structure



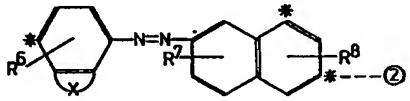

wherein

$R^1$ is a sulphonated group derived from anthroquinone, a substituted anthroquinine, an aromatic azo group or a thalocyanine compound and $R^2$ is either (a) an organic, normally a sulphonated aromatic group, especially a sulphonated phenyl group, or (b) a chlorine atom. Typically $R^1$ may have the structure



wherein

$R^3$, $R^4$, $R^5$ each represent a sulphonyl group or a hydrogen atom or an alkyl or amino substituted derivation of such structure. Alternatively $R_1$ may have the structure



wherein

$R^6$, $R^7$, $R^8$ are sulphonyl groups or hydrogen atoms, X may be two hydrogen atoms or a benzene ring, optionally sulphonated, and the point of attachment to the triazinyl ring may be any of points marked "*", or substituted derivatives of such a structure. However, numerous other such compounds are known. Compounds suitable for separation of human albumin include those sold under the Trade Marks Procion Red HI—8BN; Procion Red P—3BN; Procion Green H—4G; Procion Brown H—GR; Procion Blue MX—G; Procion Blue HE—RD; and especially Procion Blue H—B and Cibacron Blue 3G—A all as hereinafter described. When commercial dyes are used it may be necessary to remove wetting agents, for example, by washing with ether or acetone.

The matrix may be any support commonly used for affinity chromatography media, for example polymers and copolymers of agarose, dextrose, dextrans and amides, especially acrylamide. Glass beads or nylon matrices may also be used. Cellulose and substituted celluloses are generally unsuitable since although they bind large weights of dye, this is poorly accessible to the protein resulting in poor protein binding. Preferably the matrix is an agarose polymer or copolymer.

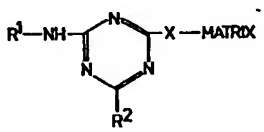
The optimum concentration of alkali metal hydroxide depends on the structure of the ligand. Thus for monochloro-triazinyl derivatives ($R^2$ = an organic group), the pH should be at least 9.5 to achieve an optimum coupling. Normally the alkali concentration should be 0.02 to 0.4, preferably 0.05 to 0.2N, although the upper limit is not particularly critical.

With dichloro-triazinyl derivatives ($R^2$ = chlorine) the alkali concentration should be about 0.002 to 0.1, preferably 0.005 to 0.01N (pH about 8 to 12.5) and the ligand binding is found to fall off quite rapidly once an optimum alkali concentration is exceeded.

The ligand-matrix coupling reaction may be conducted over a wide range of temperature, within the stability ranges of both reagents, without serious effect on the amount of ligand bound. However the mono-chloro triazinyl ligands bind only slowly so that at ambient temperatures (15—25°C) as long as 40 to 80 hours may be required for optimum reaction, and elevated temperatures of 40° to 60°C are preferred since they both speed the reaction and yield a media showing a tighter elution profile. Dichloro-triazinyl-ligands normally react in 1 to 4 hours at ambient temperatures, there appears no significant advantage in using higher temperatures. The presence of sodium chloride in the reaction slurry typically at 0.25 to 0.5 Molar, is found to facilitate dye incorporation by a common ion effect. Generally the presence of sodium chloride approximately doubles the dye binding although excessive amounts may reverse this effect.

The presence of chloride groups in the solid medium may have an adverse effect on protein-binding and hence when dichloro-ligands are used the above process should preferably be followed by a further step to convert any free chloride groups to less harmfull groups such as amine groups.

The process of the present invention permits much higher concentrations of dye to be bound to the matrix than was possible with previously known methods without cyanogen bromide, and provides protein-binding solid media comprising a protein-binding ligand containing triazinyl groups bound directly to a matrix having substantially the structure



wherein

X represents —O— or —NH— and $R^1$ and $R^2$ are as defined above.

The mixture from which the albumin is to be separated may, for example be blood plasma or serum of human, or possibly animal, origin. Alternatively it may be a separated blood protein fraction

such as Cohn fraction IV or may be washings from red cells. According to a preferred application of the invention however the albumin containing mixture is either whole blood or plasma (after separation of red cells obtained during plasmaphoresis.

Plasmaphoresis, the removal of plasma with return of the patients red cells, is now used in the treatment of several disease states. Blood cell separators are used to remove from the patient large amounts of plasma, containing one of a number of damaging substances, replacement being made with plasma from normal donors. The transfusion of whole plasma is wasteful in so far as most recipients require only a single protein, e.g. albumin. As a consequence albumin or plasma protein fraction (4.5% albumin) is used to transfuse the patient. However, this is wasteful of expensive albumin stocks.

The idea of returning to the patient his own albumin is very attractive since the plasma withdrawn in therapeutic plasmaphoresis cannot be used for normal fractionation of plasma proteins. In accordance with this aspect of the invention the affinity separation of albumin purification may be coupled into the blood cell separator, either manually or automatically, so that the patient's albumin can be removed for subsequent transfusion. To achieve this end the elution conditions should be such that the albumin solution eluted from the absorbent has a concentration approaching 4.5g%.

The albumin containing mixture should be contacted with the chromatography medium at the lowest convenient ionic strength, preferably below 0.1M although ionic strengths of 0.15M (blood plasma) and up to 0.3M may conveniently be used. Ionic strengths above 0.5M should preferably be avoided. Acid pH's give higher albumin binding, but a lower purity product due, presumably, to less specific binding. For optimum albumin recovery the pH should be 6.0 to 7.5, but for optimum purity a pH of 7 to 8 is preferred. The choice of buffer appears to have little effect on recovery. Phosphate buffers are suitable, but citrate, acetate, succinate or tris-HCl buffers may also be used. The temperature affects the binding capacity of the column, temperatures above 20°C, preferably at least 30°C, being preferable. Temperatures above 40°C are likely to degrade the albumin and hence may be undesirable. The purity of the eluted albumin does not, however, appear to be affected by temperature.

The eluting solution will normally contain a buffer the same as or more alkaline than that in the albumin-containing mixture. The eluting solution should contain at least 1mM of carboxylic acid, preferably a $C_4$ to $C_{12}$ carboxylic acid, normally in the form of an alkali metal or other salt. Octanoic (caprylic) acid is an especially preferred eluting agent since it is pharmaceutically acceptable as a stabiliser in the separated albumin. Addition of inorganic salts, preferably sodium chloride, to the eluting solution increases the rate of elution of albumin at a given carboxylic acid strength. Hence the eluting solution preferably has an ionic strength of at least 1 M NaCl or a carboxylic acid concentration of at least 4mM. Much higher carboxylic acid concentrations may be used, but may offer little advantage or even be detrimental to the properties of the separated albumin solution.

When used to enhance agglutination in blood group reference and cross match testing, the product of the present invention shows high enhancement activity with very low tendency to give false positives by rouleaux formation as experienced with bovine albumin. The enhancement may be further increased by heat treatment at above 50°, preferably about 60°C, typically for about 10 hours in the presence of the carboxylic acid.

For this purpose the carboxylic acid concentration should preferably be not more than 10mM preferably about 6mM.

The invention will now be illustrated by examples describing the separation of human albumin from mixtures containing same by methods according to the invention and by comparative methods.

The dyes used in the following examples are commercially available mono-or-di-chloro triazine reactive dyes and are identified by their trade name including the Trade Marks "Procion" and "Cibacron". The structures, and Colour Index Constitution Numbers (CICN) are listed in Table 1.

The matrices used were un-crosslinked and crosslinked (CL) Agarose gels supplied by Pharmacia Fine Chemicals, Inc. under the Trade Mark "Sepharose". Weights of agarose (except freeze-dried) are moist weights 1g being approximately equivalent to 1 ml settled volume or 40 mg or 60 mg dry weight of Sepharose 4B (or CL—4B) and 6B (or CL—6B) respectively.

TABLE 1

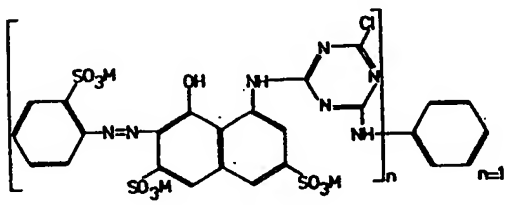
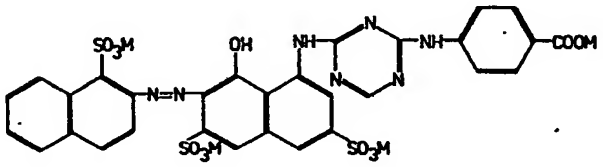
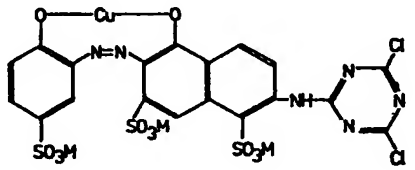
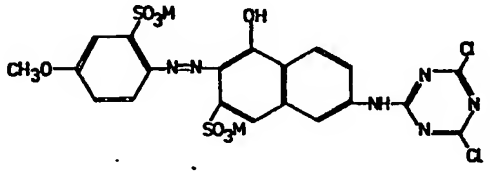
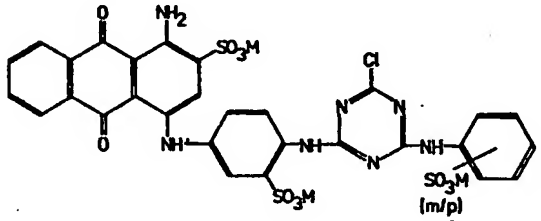
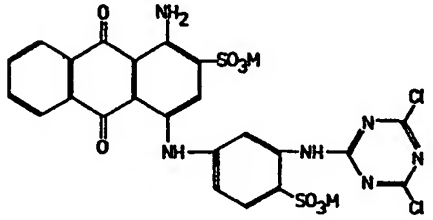
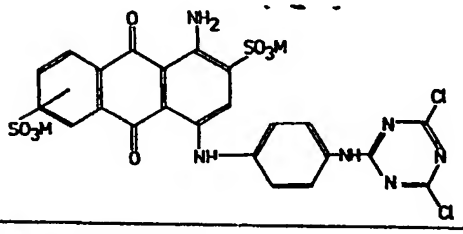
| DYE NAME | CICNo | STRUCTURAL FORMULA |
|---|---|---|
| Procion* Red H—3B | 18159 |  |
| Procion* Red HE—3B | — | As Procion Red H—3B, but n=2 (p- isomer) |

TABLE 1 (cont.)

| DYE NAME | CICNo | STRUCTURAL FORMULA |
|---|---|---|
| Procion* Red P3BN | – | Similar to Procion Red H–3B |
| Procion* Red H–8BN | – |  |
| Procion* Rubine MX–B | 17965 |  |
| Procion* Scarlet MX–G | 17908 |  |
| Cibacron* Blue 3GA (formerly F3GA) *and* Procion Blue H–B | 61211 |  |
| Blue Dextran | – | As Cibracron Blue 3GA, but-O-Dextran in place of –Cl |
| Procion* Blue MX–R | 61205 |  |
| Procion* Blue MX–3G | |  |

In each case 'M' represents a hydrogen or sodium ion
* 'Procion' and 'Cibacron' are Trade Marks.

In the examples the following standard procedures were used unless otherwise indicated. All percentage concentrations are weight/volume (i.e. g/100 ml).

Methods for immobilisation of triazine dyes

Triazine dyes are washed with acetone and ether prior to use.

Dichloro-triazine dyes (Procion MX dyes)

10g. agarose gel (either cross-linked or ordinary) is suspended in 45 ml water containing 100 mg triazine dye. Mix well and incubate at room temperature.

After 5 min. 20% NaCl (5 ml) is added to the reaction mixtures and incubation with mixing allowed to proceed for 30 minutes, at this time 0.12 ml of 5M NaOH is added to the reaction mixtures and the reaction is allowed to proceed at 22°C for 20 min. to 2 hours. On completion of reaction the adsorbents are washed with $H_2O$, 6M Urea (or 2M NaCl), $H_2O$ and suspended in 10 mM $Na_2HPO_4$—$NaH_2PO_4$, 0.15M NaCl, 0.02% sodium azide, pH 7.5. The adsorbents are stored at 4°C.

Monochloro-triazine dyes (Procion H and HE-dyes and Cibacron 3G—A)

As above except that 1.2 ml of 5M NaOH are used and the reaction is conducted either at 22°C for 72 hours or at 60°C for 2 hours (in which case cross-linked agarose is used and pre-incubated at 60°C.

Determination of bound dye

Bound dye levels are expressed as mg dye per g. (wet weight) agarose as determined from optical density at a wavelength characteristic of the dye by either

i) Difference — i.e. difference between initial O.D. and O.D. found in washings after completion of the coupling reaction, or

ii) Digestion of dye-Sepharose

a) Un-crosslinked agarose (Sepharose 2B, 4B and 6B) digested in 50% glacial acetic acid at 80°C.

b) Cross-linked agarose 1g CL-Sepharose weighed into a conical flask and 1 ml $H_2O$ added plus 2 ml HCl (concentrated). Flask rotated carefully and placed in a water bath at 40°C for 30 minutes. Allow to cool and add 50 ml 0.1M $Na_2HPO_4$—$NaH_2PO_4$, (pH 7.0) prior to adjusting pH value at 7.0 with 5M NaOH. Dilute to 100 ml with 0.1M phosphate, pH 7.0 and determine O.D at the absorption maximum.

Albumin capacity of adsorbents

A standard column (0.7 cm x 2.6 cm) is packed with 1g Cibacron-blue 3G—A-agarose and equilibrated with 10 mM $Na_2HPO_4$—$NaH_2PO_4$, 0.15M NaCl, pH 7.5. An albumin solution (100 mg in 1.5 ml equilibration buffer) is applied to the column and the column washed with equilibration buffer until no further protein is eluted (accessed by monitoring the eluant absorbance at 280 nm). Adsorbed albumin is then eluted with ether 2M NaCl in 10 mM $Na_2HPO_4$—$NaH_2PO_4$, pH 7.5 or 20 mM sodium octanoate in equilibration buffer.

Processing albumin eluate

The eluate can be concentrated using either a hollow fibre cartridge (Amicon) or a Pellicon cassette system (Millipore). Where necessary dialysis against 0.15M NaCl can be achieved using the same equipment. Albumin solutions are concentrated to either 4.5g% or 20g% and sodium octanoate is added to a concentration of 7.2mM or 32mM prior to the pasteurisation stage (10 hours at 60°C). The solutions are then sterile filtered and stored at 15°C.

Column regeneration

There is usually no need to regenerate the columns between individual chromatographic procedures. However, the column should be re-equilibrated against the appropriate running buffer prior to sample application.

Occasionally it is beneficial, in respect to maintaining flow rates and reducing back pressure, to wash the column with one of the following solutions: 6M urea, 0.1M NaOH, 5M guanidine hydrochloride or 4M potassium thiocyanate.

Comparison of coupling procedures

EXAMPLE 1 (Comparative)

Method of Bohme (J. Chromatogr. *69* (1972) 209)

2g Cibacron blue 3G—A was dissolved in $H_2O$ (45 ml) and added to 10g Sepharose CL—6B suspended in $H_2O$ (365 ml). The reaction mixture was divided into four equal volumes and mixed for 30 minutes at 60°C. NaCl (10.25g) was added to reach reaction mixture and the reaction allowed to proceed for 60 minutes. At this time the temperature was increased to 80°C and each reaction mixture was charged with 1g $Na_2CO_3$. The reaction was allowed to proceed for another 2 hours. The bound dye and albumin binding capacity were determined as described above.

EXAMPLES 2 AND 3

Example 1 was repeated using 0.4 and 0.1 g of dye.

EXAMPLES 4—9 — Present invention

10g Sepharose CL—6B was suspended in 45 ml $H_2O$ containing 2, 0.4 and 0.1 g Cibacron blue 3G—A, and mixed for 5 minutes at 60°C. Following the addition of 20% NaCl (5.0 ml) the reaction was allowed to proceed for 30 minutes when 5M NaOH (1.2 ml) was added and the reaction allowed to proceed for 2 hours at 60°C (Examples 4—6). Using identical reactants this procedure was repeated at 22°C, reaction time 69 hours (Examples 7—9). Bound dye concentrations and albumin binding capacities were determined as in Example 1.

EXAMPLES 10—14

Cyanogen bromide methods.

4.0g Freeze-dried cyanogen bromide activated agarose (Sepharose 4B-Trade Mark of Pharmacia Fine Chemicals, Inc.) was reswollen with $10^{-3}$ M HCl and washed according to the manufacturer's instructions. Bound dye concentrations and albumin binding capacities of the resulting media were determined as in Example 1.

EXAMPLE 10

2g swollen CNBr-activated agarose was suspended in 0.1M $NaHCO_3$; 0.5M NaCl, pH 9.5 (18 ml), Cibacron blue 3G—A (50 mg) was dissolved in $H_2O$ (2.0 ml) and added to the suspension. Coupling was allowed to proceed at room temperature (22°C) for 22 h using a Coulter mixer to rotate and mix the suspension. The suspension was then poured onto a sintered funnel and the dye-Sepharose complex washed with $H_2O$. After removal of uncoupled dye the dye-Sepharose was incubated for 2hr in 1M Tris-HCl, pH 9.0 then washed successively with water, 2M NaCl, and water. The washed product was suspended in 10 mM $Na_2HPO_4$—$NaH_2PO_4$; 0.15M NaCl, pH 7.5.

EXAMPLE 11

As EXAMPLE 10 except that the Cibacron blue 3G—A was omitted from the coupling reaction to determine the albumin binding due to the agarose matrix alone.

EXAMPLE 12

(Method of Travis & Pannell, U.S. Patent 4,016,149)

As EXAMPLE 10, except that 0.5M NaCl was omitted from the coupling reaction mixture. Also the incubation period in 1M Tris-HCl, pH 9.0 was omitted. The washing procedure was changed to $H_2O$; 0.1M $NaHCO_3$, pH 9.5; 6M Urea; $H_2O$; 0.05M Tris/0.5M NaCl, pH 8.0.

EXAMPLE 13

(Method of Travis & Pannell, U.S. Patent 4,016,149)

As EXAMPLE 12 using Blue Dextran (500 mg) instead of Cibacron blue 3G—A.

EXAMPLE 14

(Method of Travis & Pannell, U.S. Patent 4,016,149)

3.5g (wet weight) agarose was suspended in 20 ml $H_2O$ containing 50 mg Cibacron blue 3G—A. The reaction mixture was incubated in a shaking water bath at 80°C for 45 minutes, the pH being held to at least pH 6.0 by the addition of sodium bicarbonate. The product was washed with $H_2O$, 5M guanidine hydrochloride, $H_2O$ and suspended in 10 mM $Na_2HPO_4$—$NaH_2PO_4$, 0.15M NaCl, pH 7.5.

The results of EXAMPLES 1 to 14 are shown in Table 2.

TABLE 2

| EXAMPLE | mg dye/g agarose available for coupling | coupled | mg albumin per g agarose | bound per mg, dye |
|---|---|---|---|---|
| 1 (Bohme) | 200 | 9.41 | 24.2* | 2.6 |
| 2 ( ,, ) | 40 | 5.95 | 16.6* | 2.8 |
| 3 ( ,, ) | 10 | 2.03 | 5.5* | 2.7 |
| 4 (Invention – 60°C) | 200 | 14.3 | 40.2* | 2.8 |
| 5 ( ,, ) | 40 | 6.9 | 27.3* | 4.0 |
| 6 ( ,, ) | 10 | 2.6 | 11.4* | 4.4 |
| 7 (Invention – 22°C) | 200 | 34.5 | 42.5* | 1.2 |
| 8 ( ,, ) | 40 | 14.1 | 38.7* | 2.7 |
| 9 ( ,, ) | 10 | 5.4 | 20.6* | 3.8 |
| 10 (CNBr-activated) | 25 | 1.4 | 0.70≠ | – |
| 11 (no dye) | 0 | 0 | 0 | – |
| 12 (CNBr-activated) | 25 | 0.45 | 0.68≠ | – |
| 13 ( ,, ) | 250≠ | 46 | 9.17≠ | 0.2 |
| 14 ($NaHCO_3$) | 14 | 0.8 | 0.65≠ | – |

≠Includes dextran

* Sepharose CL 6B
≠ Sepharose 4B

"Sepharose" is a Trade Mark.

EXAMPLE 15
(Present invention — 60°C)

40g cross-linked agarose (Sepharose CL—6B) was suspended in 180 ml $H_2O$ containing 400 mg Cibacron blue 3G—A. The suspension was mixed at 60°C for 5 minutes when 20 ml of 20% NaCl was added. After a further 30 mins. 4.8 ml of 5M NaOH was added and the reaction allowed to proceed at 60°C for 27 hours taking samples at intervals. Bound dye concentrations and albumin binding capacities are shown in Table 3.

EXAMPLE 16

Example 15 was repeated (on one tenth scale) except that further portions of Cibacron blue (40mg in 2.0 water) were added at hourly intervals over the 5 hours reaction time. Results are shown in Table 3.

TABLE 3

| Reaction Time (Hours) | EXAMPLE 15 | | | EXAMPLE 16 | | | |
|---|---|---|---|---|---|---|---|
| | dye bound (mg/g) | mg albumin per g agarose | mg dye | dye added mg/g | dye bound | mg albumin per g agarose | mg dye |
| 1 | 2.5 | 9.63 | 3.6 | 10 | 2.37 | 9.7 | 4.1 |
| 2 | 3.13 | 10.1 | 3.2 | 20 | 4.35 | 16.2 | 3.7 |
| 3 | — | — | — | 30 | 6.04 | 20.6 | 3.4 |
| 4 | 2.52 | 9.7 | 3.8 | 40 | 8.00 | 23.5 | 2.9 |
| 5 | — | — | — | 50 | 9.31 | 26.0 | 2.8 |
| 21½ | 1.37 | 4.7 | 3.4 | — | — | — | — |
| 27 | 1.02 | 3.7 | 3.6 | — | — | — | — |

EXAMPLE 17

Effect of triazine dye on albumin absorption.

Triazine dye adsorbent media were prepared according to the procedures given in general methods using Sepharose 4B as the matrix. H— and HE— dyes were coupled at room temperature, reaction time 72 hours.

Albumin capacity was determined by the procedure given in general methods: 100mg albumin in 10 mM $Na_2HPO_4$—$NaH_2PO_4$, pH 7.0 (1.5 ml) was applied to each adsorbent. Results are shown in Table 4.

TABLE 4

| | mg. albumin bound/ g. medium | mg. albumin bound/ mg. dye immobilised |
|---|---|---|
| Red HE-3B | 4.2 | 1.8 |
| ,, HE-7B | 6.6 | 2.7 |
| ,, H-8BN | 5.7 | 4.4 |
| ,, MX-8B | 2.1 | 0.5 |
| ,, H-3B | 3.2 | 1.7 |
| ,, P-3BN | 4.3 | 4.5 |
| Rubine MX-B | 2.3 | 1.4 |
| Scarlet MX-G | 2.8 | 0.6 |
| Green HE-4BD | 4.9 | 2.6 |
| ,, H-4G | 3.0 | 13.5 |
| Brown H-GR | 5.12 | 4.4 |
| ,, MX-5BR | 3.9 | 0.9 |
| Blue MX-R | 4.8 | 1.0 |
| ,, MX-3G | 3.8 | 1.4 |
| ,, MX-G | 7.04 | 3.2 |

TABLE 4 (Continued)

| | mg. albumin bound / g. medium | mg. albumin bound / mg. dye immobilised |
|---|---|---|
| Blue MX-4GD | 5.0 | 1.9 |
| ,, H-B | 13.9 | 6.0 |
| ,, 3G-A | 14.8 | 5.5 |
| ,, HE-RD | 13.1 | 3.5 |

EFFECT OF IONIC STRENGTH ON ALBUMIN ABSORPTION

EXAMPLE 18

A column (0.7 cm x 2.6 cm) was packed with 1g Cibacron blue 3G—A-Sepharose CL—6B (17.7 mg.dye/g. Sepharose CL—6B) and equilibrated with 10mM $Na_2HPO_4$—$NaH_2PO_4$, pH 7.5. An albumin solution (100 mg in 1.5 ml equilibration buffer) was applied to the column and the column washed with equilibration buffer until no further albumin was eluted (accessed by monitoring the eluant absorbance at 280 nm). Adsorbed albumin was eluted with a 2M NaCl in 10 mM $Na\ H_2PO_4$—$Na_2\ HPO_4$ (pH 7.5). Flow rate was 18 ml/h throughout. Results are shown in Table 5.

EXAMPLES 19—27

The process was repeated using a similar equilibration buffer, but containing increasing concentrations of sodium chloride. Results are shown in Table 5.

TABLE 5

| Example | NaCl conc added | mg albumin/g. adsorbent | | % Recovery |
|---|---|---|---|---|
| | | Adsorbed | Desorbed | |
| 18 | 0M | 17 | 18.3 | 107 |
| 19 | 0.05M | 18.8 | 14.2 | 76 |
| 20 | 0.1M | 15.7 | 12.6 | 80 |
| 21 | 0.15M | 12.8 | 10.4 | 81 |
| 22 | 0.2M | 10.2 | 9.3 | 91 |
| 23 | 0.3M | 11.9 | 8.2 | 69 |
| 24 | 0.4M | 9.6 | 5.0 | 52 |
| 25 | 0.5M | 4.5 | 2.6 | 58 |
| 26 | 0.8M | 4.7 | 0.7 | 15 |
| 27 | 1.2M | 0.8 | 0.1 | 13 |

EFFECT OF pH ON ALBUMIN ABSORPTION AND DESORPTION

EXAMPLES 28—44

The process of Example 18 was repeated using a variety of equilibrating buffers (all of constant ionic strength-conductivity 1.8 mm hos) and eluting with 2M NaCl in the same buffers. Results are shown in Table 6.

TABLE 6

| Example | Buffer | | mg albumin/g. adsorbent | | % Recovery |
|---|---|---|---|---|---|
| | | | Adsorbed | Desorbed | |
| 28 | Sodium citrate-citric acid | pH 4.0 | 20.06 | 10.59 | 53 |
| 29 | ,, ,, ,, | pH 4.5 | 22.54 | 13.91 | 62 |
| 30 | ,, ,, ,, | pH 5.0 | 20.59 | 14.35 | 70 |
| 31 | ,, ,, ,, | pH 5.5 | 21.62 | 18.01 | 83 |
| 32 | ,, ,, ,, | pH 6.0 | 19.27 | 18.19 | 94 |
| 33 | $NaH_2PO_4$ – NaOH | pH 6.0 | 22.88 | 21.72 | 95 |
| 34 | Sodium acetate-acetic acid | pH 6.0 | 23.44 | 22.61 | 97 |
| 35 | Succinic acid – NaOH | pH 6.0 | 22.6 | 20.36 | 90 |
| 36 | $Na_2HPO_4$ – citric acid | pH 6.0 | 19.31 | 19.1 | 99 |
| 37 | $Na_2HPO_4$ – $NaH_2PO_4$ | pH 6.5 | 18.03 | 17.2 | 96 |
| 38 | ,, ,, | pH 7.0 | 16.4 | 16.9 | 103 |
| 39 | ,, ,, | pH 7.5 | 15.25 | 13.9 | 92 |
| 40 | ,, ,, | pH 8.0 | 14.0 | 12.2 | 87 |
| 41 | Tris-HCl | pH 7.5 | 17.0 | 15.3 | 90 |
| 42 | ,, | pH 8.0 | 15.8 | 12.8 | 81 |
| 43 | ,, | pH 8.5 | 14.2 | 10.8 | 76 |
| 44 | ,, | pH 9.0 | 10.0 | 8.2 | 82 |

EFFECT OF TEMPERATURE ON ALBUMIN ADSORPTION

EXAMPLES 45—48 (A and B)

A column (1.6cm x 30cm) was packed with 13g Procion blue H—B Sepharose CL—6B (9.3mg dye/g Sepharose CL—6B) and equilibrated with 10mM $Na_2HPO_4$—$NaH_2PO_4$, 0.15M NaCl, pH 7.5 at the appropriate temperature. An albumin solution (2.0g in 32 ml equilibration buffer) or Cohn Fraction IV (100 ml, containing 13mg/ml albumin) was applied to the column and the column washed with equilibration buffer until no further protein was eluted from the column (assessed by monitoring the eluant absorbance at 280mm). Adsorbed albumin was eluted with 40mM sodium octoate in 10mM $NaH_2PO_4$—$Na_2HPO_4$, 0.15M NaCl (pH 7.5) at the appropriate temperature. Flow rate was 50 ml/h throughout.

As shown in Table 7 the adsorbent capacity shows a strong temperature dependence, purity of eluted albumin was independent of temperature.

TABLE 7

| Example | Temperature °C | Capacity (mg albumin/g. adsorbent | |
|---|---|---|---|
| | | A — Albumin Loading | B — Fraction IV Loading |
| 45 | 4.5 | 16.8 | 15.5 |
| 46 | 24 | 26.5 | 26.9 |
| 47 | 30 | 31.2 | 30.2 |
| 48 | 37 | 34.3 | 33.1 |

EFFECT OF ELUANT ON ALBUMIN ELUTION

EXAMPLES 49 TO 61

Albumin was adsorbed onto a series of 24 x 1.5cm columns loaded with the medium of Example 21, using 10 mM $Na_2NPO_4$—$NaH_2PO_4$; 0.15M NaCl buffer (pH 7.5) and eluted at 46 ml/h using a series of eluants in 10 mM $Na_2HPO_4$—$NaH_2PO_4$ buffer. The volume (Ve) of eluant required to elute 95% of the applied albumin was noted in each case. Results are shown in Table 8.

TABLE 8

| Example | Eluant | Ve |
|---|---|---|
| 49 | 2M NaCl/10% Ethanol, pH 8.5 | 52 |
| 50 | 2M NaCl pH 8.5 | 48 |
| 51 | 2M NaCl pH 7.0 | 46 |
| 52 | 2M NaCl pH 8.0 | 44 |
| 53 | 2M NaCl pH 6.5 | 44 |
| 54 | 2M NaCl/20% Glycerol, pH 7.5 | 44 |
| 55 | 0.15M NaCl; 7mM Sodium Octanoate pH 7.5 | 44 |
| 56 | 2M NaCl/10% Ethanol pH 7.5 | 42 |
| 57 | 7mM Sodium octanoate, pH 7.5 | 36 |
| 58 | 3M NaCl, pH 7.5 | 30 |
| 59 | 2M NaCl, 4mM sodium octanoate, pH 7.5 | 26 |
| 60 | 0.15M NaCl, 20 mM sodium octanoate pH 7.5 | 21 |
| 61 | 2M NaCl, 7mM sodium octanoate, pH 7.5 | 20 |

EXAMPLES 62—69

The procedure of Example 21 was repeated except that the albumin was eluted by a linear concentration gradient of various carboxylic acids in the equilibration buffer (20 ml total volume). Results are shown in Table 9.

TABLE 9

| Example | Eluant | Gradient limits | % Recovery Albumin | Eluant eluting concentration | |
|---|---|---|---|---|---|
| | | | | Peak | Complete |
| 62 | Sodium formate | 0–2.0M | 53 | 0.65M | >2.0M |
| 63 | ,, acetate | 0–2.0M | 48 | 0.45M | >2.0M |
| 64 | ,, propionate | 0–2.0M | 74 | 0.4M | 2.0M |
| 65 | ,, butyrate | 0–2.0M | 96 | 0.25M | 1.0M |
| 66 | ,, valerate | 0–1.0M | 96 | 0.18M | 0.6M |
| 67 | ,, hexanoate | 0–0.25M | 88 | 0.06M | 0.25M |
| 68 | ,, octanoate | 0–0.25M | 100 | <0.05M | 0.15M |
| 69 | ,, decanoate | 0–0.25M | 100 | <0.05M | 0.15M |

*Purification of albumin from mixtures using triazine dye-adsorbents.*

EXAMPLES 70 TO 72

Cohn Fraction IV

Cohn Fraction IV paste (1 kg) was suspended in $H_2$ (4 l), prechilled to 4°C. The suspension was agitated for 5 min. using a Silverson mixer and then brought to pH 7.5 by the addition of 4N NaOH. After further mixing the suspension was left overnight at 4°C to allow insoluble material to separate by flotation. The solution was filtered through asbestos pads (Carlson Ford Grade BK9 and EKS) and either used immediately or stored frozen at –25°C.

EXAMPLE 70

A 0.7 x 2.6 cm column was packed with 1g Procion Brown H—GR/Sepharose 6B (1.16 mg/g). The adsorbent was equilibrated with 10mM $Na_2HPO_4$—$NaH_2PO_4$; 0.15M NaCl, pH 7.5. Fraction IV solution (2.5 ml containing 35 mg albumin) was applied to the column and the adsorbent washed with equilibration buffer until no further protein was detected in the eluate. Protein content was assessed by monitoring the absorbance at 280 nm. Albumin was eluted with 20mM sodium octanoate prepared in the equilibration buffer. Albumin containing fractions were concentrated by ultrafiltration and the purity accessed by electrophoresis. Albumin recovery and purity was in excess of 95%.

EXAMPLE 71

The procedure of Example 70 was repeated using Procion blue MX—R/Sepharose 6B (4.9 mg/g) as the adsorbent. Albumin elution was achieved with 2M NaCl, recovery and purity in excess of 90%.

EXAMPLE 72

The procedure of Example 70 was repeated using Procion blue HE—RD/Sepharose 6B (3.74 mg/g) as the adsorbent. Albumin recovery approached 100%, purity was assessed at greater than 90%.

EXAMPLE 73

A column (4.4 cm x 30 cm) was packed with Cibacron blue 3G—A-Sepharose CL—6B (200g) and equilibrated with 0.15M NaCl, 10mM $Na_2HPO_4$—$NaH_2PO_4$ — pH 7.5, flow rate 500 ml/h. 100 ml human plasma was applied to the column and the column washed with equilibration buffer until the absorbance at 280 nm approached zero. Albumin elution was achieved with 20mM sodium octanoate in equilibration buffer. On completion of albumin desorption the column is re-equilibrated for another loading of plasma.

Albumin recovery was in excess of 95%. Albumin purity by quantitative electrophoresis was 99%; monomer content 95%.

EXAMPLES 74—79

The procedure of Example 73 was repeated using 3M NaCl in 10mM $Na_2HPO_4$—$NaH_2PO_4$, pH 7.5

as the eluant. The product was concentrated to 5g/100ml and diafiltrated against 0.15M NaCl, 10mM $Na_2HPO_4$—$NaH_2PO_4$, pH 7.5. The product was concentrated to 25g albumin per 100 ml and heat treated to 60°C for 10 hours in the presence, of 6, 10, 20, 25, 30 and 35mM sodium octanoate. The products were compared to a non-heat treated, sodium octanoate free solution of the same albumin (also 25g/100 ml) for agglutination enhancement with a variety of antibody/antigen combinations. Significantly greater enhancement was observed with the 10mM and especially 6mM samples.

CLAIMS

1. A process for separating albumin from a mixture containing same comprising contacting said mixture with a suspension of an affinity chromatography medium to which the albumin will bind, washing said medium to remove unbound components of said mixture and eluting the albumin from said medium by washing with an eluting solution containing a carboxylic acid preferably having 3 to 14 carbon atoms, wherein the affinity chromatography medium is an immobilised triazine dye produced by reacting a protein-binding ligand material containing chlorotriazinyl groups with an aqueous suspension of a non-cellulosic matrix containing free hydroxy or amino groups in the presence of an alkali metal hydroxide at a pH of at least 8 and subsequently washing the resulting solid medium to remove unreacted dye.

2. A process according to claim 1 wherein the mixture containing albumin is whole blood or blood plasma.

3. A process according to claim 1 wherein the said mixture has an ionic strength of less than 0.1 Molar.

4. A process according to any one of claims 1 to 3 wherein the said mixture has a pH of 6.0 to 7.5.

5. A process according to any one of claims 1 to 3 wherein the said mixture has a pH of 7 to 8.

6. A process according to any preceding claim wherein the said mixture contains a phosphate buffer.

7. A process according to any preceding claim wherein the said mixture is contacted with the said suspension at a temperature above 20°C.

8. A process according to claim 7 wherein the temperature is 30—40°C.

9. A process according to any preceding claim wherein the eluting solution has a pH at least as high as that of the mixture containing albumin.

10. A process according to any preceding claim wherein the eluting solution contains at least 1 mM of the carboxylic acid.

11. A process according to claim 10 wherein the eluting solution contains at least 4 mM of the carboxylic acid.

12. A process according to claim 11 wherein the eluting solution contains not more than 10 mM of the carboxylic acid.

13. A process according to any one of claims 9 to 12 wherein the eluting solution contains inorganic salts.

14. A process according to claim 13 wherein the eluting solution has an ionic strength of at least 1 M NaCl.

15. A process according to any preceding claim wherein the carboxylic acid has 4 to 12 carbon atoms.

16. A process according to claim 15 wherein the carboxylic acid is octanoic acid.

17. A process according to any preceding claim wherein the carboxylic acid is present as an alkali metal salt.

18. A process according to any preceding claim wherein the separated albumin is subsequently heat treated to at least 50°C in the presence of a carboxylic acid having 3 to 12 carbon atoms.

19. A process according to claim 18 wherein the carboxylic acid is octanoic acid.

20. A process according to either claim 18 or 19 for producing albumin for serological testing wherein the concentration of the carboxylic acid is not more than 10mM.

21. A process according to any one of claims 18—20 wherein the albumin is heated to about 60°C.

22. A process for separating albumin substantially as hereinbefore described with reference to the examples.

23. Albumin separated by a process according to any preceding claim.

24. A process according to any one of claims 1—17 wherein the separated albumin is subsequently returned to the patient from whom the mixture containing albumin was derived.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1981. Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.